Sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPases, myogenic regulatory factors and myostatin in the regeneration of rat skeletal muscles

Summary of Ph.D. Thesis

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2000

1. Introduction

In recent years further steps have been made for the understanding of the time- and stage-dependent pattern of gene expression in skeletal muscle development, however, the molecular control is not completely understood yet. Since skeletal muscle regeneration recapitulates many of the principal events of embryonic development, it could be a good model for studying the regulatory mechanisms of muscle differentiation. On the other hand, the phenomenon of regeneration is clinically important since it is a part of the pathology of several muscle diseases, *e.g.* Duchenne-type hereditary dystrophies, of which proper therapy has not been found yet. As a potential therapeutical approach, activated myoblasts have been transferred into dystrophic muscles in order to improve their regeneration capacity. Thus, it becomes of interest to learn ways of modulating the outcome of regeneration.

In adult muscle the quiescent satellite cells are the main sources of regeneration located between the sarcolemma and the basal lamina of myofibers. Once the muscle is damaged these myogenic precursor cells (mpc) are activated, re-enter the cell cycle and result in active, proliferating myoblasts, most of which fuse to primitive, multinucleated myotubes. Myotubes characterized by central nuclei become larger by incorporating new myoblasts and differentiate further to mature myofibers by expressing muscle-specific proteins. One of these proteins is the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which pumps Ca^{2+} from the sarcoplasm into the sarcoplasmic reticulum (SR) essential to the relaxation of skeletal muscle. Three types of SERCA genes are known. The SERCA1 gene shows a developmental stage-dependent expression of the neonatal (SERCA1b) and the adult form (SERCA1a) in fast-twitch skeletal muscles. The SERCA2 gene is expressed virtually in every cell and can be spliced in a tissue-specific manner into the SERCA2a protein characteristic to slow-twitch skeletal muscle, cardiac muscle and into the "housekeeping" SERCA2b protein expressed in smooth muscle, neural and many other tissues. The SERCA3 gene also has splice-variants found in different non-muscle cells. The expression pattern of the SERCAs has already been described during the development of skeletal muscles, however, less is known about their expression in regeneration. It is also far from understanding, how the regeneration process itself can be regulated. The four members of the myogenic regulatory factor (MRF) family: myoD, myf-5, myogenin and MRF4 play a pivotal role in myogenic

differentiation as they were identified on the basis of their capacity to convert non-muscle cells to myoblasts. All of these transcription factors possess a similar DNA-binding domain through which they can bind to a specific DNA-region influencing the muscle-specific gene transcription. Their expression shows a unique spatial and temporal pattern during myogenesis, *i.e.* myoD and myf-5 are expressed first determining the myogenic fate, while myogenin and MRF4 appears later after the proliferating myoblasts have withdrawn from the cell cycle and fused into myotubes. Many factors proved to affect myoblast proliferation/differentiation through influencing the expression of the different MRFs. Innervation, which is indispensible for normal muscle development after myoblast fusion and for determining the muscle-type, down-regulates the levels of myoD and myogenin, while denervation has the opposite effect. However, little is known about MRF expression during the regeneration of different types of adult muscle, which also includes changes of the muscle-nerve contact.

The regulation of skeletal muscle development/regeneration should also involve the control of the mass of individual muscles and their relation to the whole body mass. A new, muscle-specific growth factor called myostatin has been reported to be an important negative regulator of muscle growth and development. Myostatin knock-out mice are significantly larger than wild-type animals mainly due to an increase in muscle mass, resulting from a combination of cell hyperplasia and hypertrophy. Moreover, some breeds of cattle characterized by increased muscle mass (double-muscling), *e.g.* Belgian Blue and Piedmontese, have also mutation in the myostatin locus suggesting the same inhibitory role for myostatin in different species. It is not clarified yet, whether myostatin has a role in different adaption reactions of adult muscle like hypertrophy/atrophy or regeneration.

There are several *in vivo* experimental models to study skeletal muscle regeneration. A well-characterized snake venom called notexin causes a fast and extensive myofiber-necrosis accompanied by inflammatory cell response. However, the toxin keeps satellite cells, blood supply, extracellular matrix and nerve-endings relatively intact, which allows an effective regeneration within 28 days.

2. Objectives

In an earlier phase of our work we characterized the morphological events of myogenic cell proliferation and differentiation in notexin-induced regeneration, including the study of the formation of new muscle-nerve contacts (motor endplates).

Based on these data the general aim of the present study was to describe some important aspects of the muscle-specific gene expression in skeletal muscle regeneration, moreover, to find the similarities and the differences between the molecular regulation of regeneration and myogenesis.

The experimental objectives could be summerized in two points:

1. To describe the time-dependent expression of SERCA isoforms (SERCA1a/1b, SERCA2a), MRF family members (myoD, myf-5, myogenin, MRF4) and myostatin during the regeneration process, and relate them to the known morphological changes.
2. To explore whether there are differences in the expression of the above mentioned factors in a slow-twitch (m. soleus) and a fast-twitch (m. extensor digitorum longus, EDL) muscle regenerating from notexin-induced necrosis.

3. Materials and methods

Induction of regeneration, animal treatment

Wistar rats (300-360g) were used for the experiments. The rats were narcotized and the slow-type soleus or the fast-type EDL muscles were directly injected with 20 μg or 60 μg of notexin, respectively, in physiological salt solution. At 1, 3, 5, 7, 10, 21 and 28 days after injection the entire soleus or EDL muscles were dissected and the animals were sacrificed by an overdose of sodium pentobarbital. For each time point at least 3 animals were treated. Control samples were taken from the contralateral muscles injected with 0.9% NaCl. The dissected muscles were frozen in isopentane cooled by liquid nitrogen and kept at -70 ^{o}C. From the central part of each frozen muscle, sections were stained with hematoxylin-eosin to monitor the necrosis and the subsequent regeneration.

mRNA analysis: reverse transcription-based polimerase chain reaction (RT-PCR)

Total RNA was isolated from control and regenerating soleus and EDL muscles by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi 1987). The RNA pellet was dissolved in RNAse-free distilled water and RNA concentration and its purity was measured spectrophotometrically. 2 μg of total RNA was subjected to an oligo(dT)-primed first strand cDNA-synthesis. The linearity of the cDNA synthesis was confirmed by the incorporation of [α-^{32}P]dCTP and found to be linear in the range of 30-3000 ng input RNA. PCR reactions amplifying MRFs (myoD, myogenin, myf-5 and MRF4), the slow-type SERCA2a, myostatin and the internal control of the amplification, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were carried out under optimized conditions for each primer-pair. To measure the relative SERCA1a/SERCA1b ratios we used primers that encompass an optional exon, which is removed from the fast neonatal SERCA1b amplifying a shorter transcript, while retained in the adult SERCA1a mRNA, thus making the separation possible based on their size. The number of PCR cycles was carefully adjusted to avoid saturation of the amplification system. The quantification of the amplified products was done by labelling with [α-^{32}P]dCTP in two additional PCR cycles, followed by electrophoretic separation and phosphorimage analysis on a

PhosphorImager model 425/ STORM model 840 (Molecular Dynamics). For statistical analysis, the Student t-test or the Newman-Keuls test were used.

SERCA protein analysis

-Preparation of fragmented muscle membranes and Western blotting

The soleus and EDL muscles were finely minced and homogenized in 2.5 ml ice-cold 0.25M sucrose, 5mM HEPES pH 7.5. After centrifugation the pellets, which represented the combined mitochondrial and microsomal fractions, were washed in homogenization buffer and resuspended in 0.25 M sucrose. 5 µl of the suspensions were subjected to electrophoresis on a Laemmli-type of 7.5% SDS PAGE and then electroblotted onto immobilon-P nylon membrane (Millipore). After quenching the membrane the SERCA1 proteins were detected by a 1:10 dilution of culture supernatant of the A3 mouse anti-SERCA1 monoclonal antibody, while the SERCA2a proteins by a 1:500 dilution of SERCA2a-specific rabbit antisera R-15. After incubating with a peroxidase-conjugated secondary antibody (1:1000) nickel-enhanced diaminobenzidine (DAB) staining were used for visualisation of the immuncomplexes. Quantitification was performed by densitometric scanning. Amounts of samples were always applied within the linear range of the assay as determined by a dilution series of the sarcoplasmic reticulum fraction. Controls to ascertain specifity of the SERCA antibodies were also performed. Three protein homogenates were analyzed for every time point studied and the Student t-test or the Newman-Keuls test were used.

-Immunohistochemistry

Soleus and EDL muscles were cryoprotected with 20% sucrose and frozen in isopentane cooled with boiling liquid nitrogen. Serial cryosections of 20 µm thickness were cut and processed for immunostaining. Sections were incubated in blocking reagent (1% BSA and 10% normal rabbit or goat serum in PBS) followed by an overnight incubation with the SERCA1 or SERCA2a-specific antibody and finally with the peroxidase-conjugated secondary antibody. The immuncomplexes were visualized by DAB staining of peroxidase activity. Control sections were immunostained in the absence of the primary antibody. Images of the serial sections stained with the SERCA antibodies was compared using Global Vision System software (Analis Belgium, Version 1.2, Namur, Belgium).

4. Results and Discussion

Morphological studies indicated that in soleus muscle. complete necrosis could be achieved as early as by day 1 after toxin administration, while in EDL surviving fibers still existed on day 3. In soleus, most of the activated, dividing mononucleated cells filled up the space of the necrotized fibers by day 3 followed by their fusion into multinucleated myotubes from day 5 on. In parallel with myotube formation new motor end-plates were formed replacing the destroyed ones showing matured morphology by day 7 in soleus, at the time when the primitive myofibers started to differentiate and grow in size. The time-course of necrosis and subsequent regeneration was slower (with about 2 days) in EDL than in soleus muscle, which argues in favour of the higher resistance of the fast fibers to the toxin. After 28 days of regeneration both types of muscle showed similar morphology to the normal ones except that several myonuclei were still in the center of myofibers instead of being localized at the periphery, close to the sarcolemma.

Transcript levels of the myogenic regulatory factors in regenerating rat soleus and EDL muscles

The levels of mRNA for the early-acting myogenic factors (myoD and myf-5) showed quite similar time-courses in the regenerating soleus and EDL muscles. On the first day after notexin-treatment the myoD mRNA levels increased dramatically in both muscles, probably marking the activation of satellite cells and the beginning of regeneration. The comparable time-course was particularly remarkable because the complete necrosis occurred later in EDL than in soleus, suggesting that satellite cells of different types of muscles responded in a similar way, but unknown factors may have delayed the necrosis and the regeneration in the fast-glycolytic fibers. In contrast to myoD, the mRNA level of myf-5 dropped to levels below the detection limit on the first day. This suggests that myf-5 is regulated differently from myoD in the regenerating muscle, which is supported by the fact, that the transcript level of myf-5 increased in mice lacking a functional myoD gene during muscle development.

The transcript levels of the late-acting myogenic regulatory factors disappeared from soleus on day 1, but remained normal in EDL. This again probably reflects the difference in the time-course of necrosis of the two muscles, *i.e.* the mRNAs of the late MRFs disappear together with the muscle fibers in soleus, while they persist in the still surviving fibers in

EDL. On day 3, when myoblasts were already activated in soleus, the mRNA levels of both myogenin and MRF4 increased. However, in EDL, only the transcript of myogenin increased, probably reflecting the gradual replacement of the necrotizing fibers by the new myoblasts and myotubes, whereas the mRNA of MRF4 was maintained at the normal level.

It was shown that the transcription of myoD and myogenin is induced by denervation and repressed by neural stimulation. In line with this hypothesis, the levels of myoD and myogenin mRNAs declined in the later stages of regeneration for both types of muscle. A similar change was observed for myf-5, but not for MRF4 which remained relatively constant during the second half of regeneration. After 4 weeks the mRNA levels for each of the myogenic factors returned to nearly normal levels. In conclusion, we found that the order of activation of the MRFs: myoD, myf-5/myogenin, MRF4 followed a similar pattern to that during early muscle development except the earlier uprise of myoD than that of myf-5.

mRNA and protein levels of fast and slow SERCA variants in regenerating soleus and EDL muscles

As expected, in normal soleus the slow SERCA2a, while in normal EDL the adult fast SERCA1a transcript was the dominant isoform. At the time of necrosis (on day 1 in soleus and on day 3 in EDL) the SERCA transcripts disappeared from the muscles and their levels remained low until day 5. Then first the neonatal SERCA1b mRNA increased suggesting that it is an early molecular marker of the myotube formation in both muscles, but apparently it is preceded by the uprise of the MRFs. A similar increase of the neonatal SERCA1b isoform was also observed in normal development. After the first peak of the SERCA1b, large increases of the transcript levels of SERCA1a and SERCA2a were observed around day 7 in both muscles, exactly at the time when re-innervation of the new fibers occured. Thus, re-innervation might have induced a switch from the neonatal SERCA1b to the adult fast SERCA1a, and a few days later from SERCA1 to the slow-type SERCA2a mRNA. In soleus muscle after 3 weeks of regeneration the SERCA1a isoform was replaced by the slow SERCA2a and the SERCA2a/SERCA1a ratio reached a situation (ratio:3.9) found in normal adult soleus. However, this latter increase of the slow SERCA isoform was negligible and transient in the regenerating EDL, similar to the changes found in ontogenesis. This implies that the functional (slow-or fast-type) re-innervation highly determines the characteristic ratio

of different SERCA isoforms in the different muscles. For SERCA protein measurement the monoclonal antibody we used does not discriminate between the neonatal and the adult variants of SERCA1. This precluded the demonstration of a putative increase in the level of the neonatal SERCA1b protein, which might accompany the corresponding increase of its mRNA. Assumed that the first increase in the SERCA1 protein level must be ascribed to the neonatal SERCA1b, the changes of SERCA protein levels followed those of SERCA transcripts during the regeneration of both muscles. Therefore, SERCA1 and SERCA2 expressions are likely to be mainly pretranslationally controlled. Moreover, the general pattern of SERCA transcripts and proteins during notexin-induced regeneration recapitulates those observed during myogenesis.

Although the normal levels of SERCA proteins were restored after 28 days of regeneration, more fibers expressed the slow SERCA2a isoform, while less fibers showed the fast SERCA1 positivity in the regenerated soleus than in normal muscles. Moreover, the SERCA1 expressing fibers were found to co-express the slow and fast SERCA isoforms. These findings indicate that the regenerated soleus does not completely return to its condition prior to necrosis. Similar changes were found for myosin isoforms, which might reflect that contraction and relaxation are tightly coupled at the molecular level and that they are possibly influenced by common regulatory mechanisms. In regenerated fast-twitch EDL muscles we also found more hybrid fibers than in normal ones suggesting that the slow nerve-endings are slightly more efficient at re-innervating the regenerated fibres than the fast ones. A competition for re-innervation of regenerating fibers is also supported by the close spatial association of the slow SERCA2a expressing (type I) fibers in regenerated EDL muscles.

The exact mechanism of the regulation of SERCA-isoform expression in regeneration is not known. The uprise in the level of myogenic regulatory factors (MRFs) preceded the expression of SERCAs but in the later stages of regeneration no differences were observed between the fast-twitch EDL and the slow-twitch soleus in respect to their MRF expression. This suggests that the SERCA1 and SERCA2 levels are not directly controlled by these transcription factors.

Transcript levels of myostatin in regenerating soleus and EDL muscles

We found in accordance with other reports that normal soleus muscles contained lower level of myostatin transcript than normal EDL. The significance of its higher level in fast muscles is not known yet. Injecting the muscles with notexin, myostatin mRNA levels diminished in both types of muscles. In soleus, its transcript reappeared only on the third day and increased until reaching the maximum above the control on day 7. Then it declined gradually and returned close to normal after 28 days of regeneration. In EDL we measured similar changes, but as a difference, the initial decrease was more gradual than in soleus and also, no transient peak was seen, presumably reflecting the differences in the dynamics of regeneration between soleus and EDL. This pattern shows similarities to that found in porcine skeletal muscles during normal development. The dramatic decrease at the time of necrosis and cell proliferation does not necessarly mean that myogenic cell proliferation can not be inhibited by myostatin, since the possible up-regulation in mononucleated cells might be masked by its loss from the necrotizing fibers. This idea is supported by our finding (data not shown) that myostatin proteins did not follow their transcripts, instead, they showed high level at this early stage of regeneration. However, we certainly measured higher transcript levels in the second part of regeneration characterized by myofiber differentiation and growth, suggesting that myostatin can also influence these late events. In theory. myoblast fusion or re-innervation might affect its up-regulation *in vivo* based on their coincidence, however, *in vitro* (data not shown) neither fusion nor innervation seemed to be necessary for increasing its transcript level.

At the end of regeneration, when muscles regained their original size, myostatin transcript returned to normal levels. This time-dependent expression pattern supports its role in regulating muscle mass in regeneration.

5. Conclusions

1. We have established and characterized the morphological and biochemical aspects of the *in vivo* regeneration of a slow- and a fast-type skeletal muscle (m. soleus and m. EDL, respectively) following notexin-induced necrosis. The fast fibers of EDL showed higher resistance to the toxin resulting in a delayed and less synchronous degeneration/regeneration process than in soleus.

2. The order of activation of the muscle-specific transcription factors (myoD, myf-5, myogenin, MRF4) and of the different isoforms of SERCAs were similar to that found in muscle development suggesting that the expression of muscle-specific genes during regeneration largely recapitulates the events of myogenesis. The expression pattern of MRFs showed little, if any, differences in the different muscle-types. Similarly, both the regenerating soleus and the EDL muscles expressed the neonatal SERCA1b isoform, which therefore seems to be a reliable marker of regeneration.

3. Re-innervation profoundly changed the expression of MRFs and SERCA isoforms in accordance with the role of innervation in ontogenesis. Re-innervation seemed to be essential to form the original fast or slow character of the regenerated muscle. For some reasons, however, the slow nerve-endings were likely to be more effective in re-innervation of new fibers, which changed the fiber-type composition and the expression pattern of SERCAs.

4. The expression pattern of myostatin mRNA found in our experiments strongly suggests that myostatin, which regulates muscle growth during development, may also have a role in the regenerating muscle.

6. Acknowledgements

This work was supported by the 3-year doctoral fellowship of the Hungarian Ministry of Education; the grant MKM 273 from the Hungarian Ministry of Education; the grant OTKA T006277 from the Hungarian Scientific Research Found; the grant B-10/96 from OMFB and the grant BIL 96/15 Ministerie Vlaamse Gemeenschap, Belgium.

I greatly acknowledge to Professor László Dux *for inviting me to join to the department* as a student research worker and for providing me the possibility to resume the scientific work as a PhD student.

I am especially thankful to my promoter Dr. Ernô Zádor, who was supporting me *without sparing* his time and energy over the past few years. His guidance and encouragement was indispensible all the time.

I am greatful to Professor Frank Wuytack, who gave me the opportunity to work in his laboratory and showed me the way how to live and work abroad.

I am thankful to Dr. Gábor Rácz for his suggestions, advice and help in preparing the figures and editing the text of the Thesis.

Many thanks to Balásházy Istvánné for the skillful technical assistance.

Without my husband and my family I have not been able to face all the challenges I had to. I am especially thankful to them.

7. Publications

List of full papers related to the subject of the Thesis

I. **Mendler L**, Zádor E, Dux L and Wuytack F (1998) mRNA levels of myogenic regulatory factors in rat slow and fast muscles regenerating from notexin induced necrosis. *Neuromusc Disord* 8:533-541

II. #**Mendler L**, #Szakonyi G, #Zádor E, Görbe A, Dux L and Wuytack F (1998) Expression of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPases in the rat extensor digitorum longus (EDL) muscle regenerating from notexin-induced necrosis. *J Muscl Res Cell Mot* 19(7):777-785

# The authors contributed equally to this work

III. Zádor E, Szakonyi G, Rácz G, **Mendler L**, Ver Heyen M, Lebacq J, Dux L and Wuytack F (1998) Expression of the sarcoplasmic/endoplasmic reticulum Ca^{2+} transport ATPase protein isoforms during regeneration from notexin-induced necrosis of rat soleus muscle. *Acta Histochem* 100(4):355-369

IV. Zádor E, **Mendler L**, Ver Heyen M, Dux L and Wuytack F (1996) Changes in mRNA levels of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} ATPase isoforms in the rat soleus muscle regenerating from notexin-induced necrosis. *Biochem J* 320:107-113

V. **Mendler L**, Zádor E, Ver Heyen M, Dux L and Wuytack F (2000) Myostatin levels in regenerating rat muscles and in myogenic cell cultures. *J Muscl Res Cell Mot* (in press)

Other publications:

- 8 abstracts in international journals or journal supplements
- 14 abstracts in conference proceedings